TLR-signaling, from HBeAg-positive HepG2. Expression profiling showed more than twofold inhibition of nine genes compared to that of HBeAg-negative HepG2 cells (TLR7, LY96, RIPK2, NFκB1, TNF, IL-6, IL-8, IFN-β, and MAP3K1) (Fig. 2A). To confirm these results, real-time PCR was performed. All of these genes except NF-κB1 were significantly downregulated in HBeAg-positive cells compared to HBeAg-negative cells. All of these genes have important roles in the immune response and activation of transcription (Fig. 2B).

### *Effects of HBeAg on NF-κB activation*

Next, we assessed the mechanisms by which HBeAg affects cytokine and IFN production. HBV activates NF-κB, a major player in innate immune responses to viral infections (19). Therefore, we postulated that HBeAg inhibition of the activation of NF-κB might result in the inhibition of cytokine and IFN production, and the subsequent escape of an antiviral response. To test this assumption, we expressed luciferase reporter protein under the control of an NF-κB-dependent promoter in HBeAg-positive or HBeAg-negative HepG2 with or without TNF-α or LPS stimulation (Fig. 3A–E). As expected, HBeAg inhibited NF-κB promoter activity in HBeAg-positive HepG2 cells (Fig. 3A–C; $p < 0.001$ with no drug [n = 3]; $p = 0.004$ with LPS [n = 3]; $p = 0.0075$ with TNF [n = 3]). These results were also confirmed by the transient HBeAg-expression assay in HepG2 ($p = 0.038$, n = 3) and Huh7 cells ($p = 0.0091$, n = 3) (Fig. 3D and E). These findings suggest that HBeAg may affect cytokine production, at least in part, through NF-κB.

### *Effects of HBeAg on IFN-β activation*

NF-κB stimulation leads to the expression of multiple cellular factors, including IFN-β, a central player in the innate immune response that is activated upon virus infection. In order to ascertain whether HBeAg inhibits IFN-β-promoters, we performed experiments using IFN-β-promoter luciferase reporter, essentially as described in the previous section. That is, we used the luciferase gene under the control of an IFN-β-stimulated promoter, and examined its expression in HBeAg-positive and HBeAg-negative HepG2 cells. HBeAg inhibited IFN-β-stimulated promoter activity in HBeAg-positive HepG2 cells with ($p < 0.001$, n = 3), or without poly(I-C) ($p < 0.001$, n = 3) (Fig. 3F and G). We also confirmed these results by transient transfection experiments with HepG2 ($p = 0.0011$, n = 3), and with Huh7 ($p = 0.0081$, n = 3) (Fig. 3H and I). These results demonstrated that HBeAg inhibits both NF-κB- and IFN-β-signaling pathways in hepatocytes.

### *Cell culture fluid from HBeAg-positive HepG2 enhanced HCV subgenomic RNA replication*

To confirm the function of IFN production of these cell lines, we examined whether conditioned media from HBeAg-positive or HBeAg-negative HepG2 cells would cause any differences in HCV subgenomic RNA replication, which is IFN-sensitive replication (14), as it has been reported that there are no direct interactions between HBV and HCV replication in cell culture models and in a mouse study (1,9,11). Cell culture fluid from HBeAg-positive HepG2 cells enhanced HCV subgenomic RNA replication, more than that from HBeAg-negative HepG2 cells (Fig. 3J; $p = 0.0014$, n = 3), suggesting that HBeAg-expressing HepG2 cells contain less IFN than do HBeAg-negative cells, and that conditioned medium from HBeAg-positive HepG2 cells contains less IFN than that from HBeAg-negative cells. In this system, when we treated cells with 0, 1, 10, 100, and 1000 U/mL IFN-α, HCV subgenomic RNA levels were 100%, 57%, 39%, 28%, and 25%, respectively. We estimated that conditioned medium from HBeAg-negative HepG2 cells was equal to ~10 IU/mL IFN-α. Our results showed that HBeAg inhibits IFN production in cell culture medium.

Since the NF-κB target gene IL-6 has also been implicated in hepatitis B pathogenesis (30), the modulation of IL-6 involved in innate signaling by HBeAg was also verified at the protein level by ELISA. Our results demonstrated that IL-6 expression was downregulated in HBeAg-positive HepG2 cells (36.6 ± 30.1 pg/mL; 0 ± 0 pg/mL in conditioned medium from HepG2 control cells; 324.2 ± 15 pg/mL in conditioned medium from HBeAg-negative HepG2 cells). The concentration of IL-6 from HBeAg-positive HepG2 cells was significantly lower than that from HBeAg-negative HepG2 cells ($p = 0.00012$, n = 3).

## Discussion

In this study, we investigated the regulation of HBeAg-induced suppression of IFN and cytokines in HepG2 stably expressing HBeAg protein as a model cell line. Our results demonstrated that HBeAg expression inhibits IFN and cytokine production. Transient expression of HBeAg also downregulated both NF-κB- and IFN-β-promoter activity in HepG2 or Huh7, although the mechanisms for this downregulation are unknown. In contrast to our findings, Yang *et al.* (46) observed that HBeAg activates NF-κB through IκBα degradation, and produces TNF-α and GM-CSF in the human hepatoma cell HA22T/VGH. These differences between their findings and ours may have been caused by the differences in the cell lines, and/or promoters (33). Extensive immunological studies by the Milich group (3,4,27) demonstrated that HBeAg appears more efficient at eliciting T-cell tolerance, including production of its specific cytokines IL-2 and IFN-γ, than HBV core antigen. Our observations support the immune-modulating role of HBeAg.

Locarnini *et al.* (23) used the Tet-off tetracycline gene expression system in Huh7, and revealed that core/precore expression affected gene expression, including cytokines. The system used in our present study, with HepG2 stably expressing HBeAg, supports these findings. Our results provide further direct evidence that hepatocytes exposed to HBeAg have enhanced HCV subgenomic RNA replication, and are significantly influenced in their ability to replicate. Several recent reports have also suggested that there was no evidence of direct interaction between HBV and HCV (1,9,11), although clinical studies showed interaction between HBV and HCV replication (24). It is possible that HBV might interfere with another virus by IFN or another cytokine. A cytokine response is critical for clearance of HCV, as failure to mount a potent and broad T-cell-repertoire response results in persistent HCV replication. This would explain how patients dual-infected with HBV and HCV exhibit a selective deficit of anti-HCV immunity, while demonstrating preservation of a normal immune response to unrelated antigens.

We used RT-PCR to observe the expression of TLRs 1, 3, 4, 5, 6, and 7 in HepG2 cells. We also confirmed in the present study that HepG2 has functional TLRs 3 and 4. Preiss *et al.*

(32) could not detect an NF-κB response to 1 ng/mL–1 μg/mL LPS in HepG2, whereas we could detect such a response to 10–50 μg/mL LPS (Table 2). Downregulation of TLR2 mRNA by genotype C HBV-derived HBeAg was not observed in our study, in contrast to the results of a previous study (43), in which genotype D HBV-derived clone (23) was used. Xu *et al.* (45) reported that TLR7 was suppressed in HBV infection, supporting our results. We do not know why LY96, an important molecule for TLR4, is downregulated (Fig. 2). Viruses encode proteins that target various intracellular signaling pathways, causing their constitutive or prolonged activation, resulting in increased cell proliferation and survival (41). It is well known that HBV activates the MAPK pathway (5). It is also known that RIPK2 activates the NF-κB- and IFN-β-dependent antiviral responses (8). These findings were in accordance with HBeAg's inhibition of the production of IFNs and cytokines (Fig. 2).

What is the mechanism of the downregulation of cytokine production by HBeAg? From our results (Fig. 2), HBeAg appears to interact with the TLR signaling pathway upstream of NF-κB. In LPS stimulation, we observed downregulated TLR4 in HBeAg-positive HepG2 cells (data not shown). Although we are currently investigating this issue, TLR4 might be one of the more important molecules. Precore protein also may affect intracellular signal transduction pathways. Further studies will be needed to clear up these issues.

Many viruses have evolved strategies that block the effector mechanisms induced through IFN- and/or cytokine-signaling pathways (17). Although multiple mechanisms contribute to viral persistence, the ability of the virus to evade innate immune responses is likely to be particularly important. In this report, we have demonstrated that HBeAg suppresses IFN and cytokine mRNA expression. Exploration of the novel HBeAg-inhibiting signaling pathways could lead to the development of new therapeutic strategies for persistent HBV infection.

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## Author Disclosure Statement

The authors have no conflicts with regard to financial interests. This material has not been previously reported and is not under consideration for publication elsewhere.

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＜速　報＞

# B型急性肝炎におけるHBs抗原陽性持続期間の検討

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安田　清美[1)]　鈴木　通博[3)]　小池　和彦[4)]

はじめに：Genotype AによるB型急性肝炎が全国的に急速に広がってきている．Genotype Aによる急性肝炎は慢性化しやすいことが示唆されている．しかしながら，Genotype間でのHBs抗原陽性持続期間の差異に関しては十分な検討は行われていない．今回この点に関して検討した．

対象と方法：対象は1994年から2010年にかけて首都圏3施設で経験した急性肝炎188例である．188例中HBs抗原の消失まで経過観察が可能であった88例（核酸アナログを使用した症例は含まれていない）に関しては，HBs抗原，HBe抗原，ALTを経時的に測定して経過を調べた．HBs抗原測定は各施設および各genotypeともにALT正常化までは1～2週間毎，ALT正常化後はおよそ1カ月毎に検査を行った．HBs抗原の測定はCLIA法（化学発光免疫測定法），ジェノタイプ（GT）判定はジェノタイプ特異的プローブアッセイ（スマイテストHBVジェノタイプ判定キット：ゲノムサイエンス社）を用いて行った．

結果：対象188例のうち174例でGTが決定できた．GTA 92例（53%），GTB 20例（11%），GTC 60例（34%），GTD 1例（1%），GTF 1例（1%）であった．GTAの症例の割合は1994年から徐々に増加し，2010年にはB型急性肝炎の症例の82%（9/11）がGTAによるものであった．

HBs抗原の消失まで経過を観察できた88症例におけるHBs抗原陽性持続期間を（Fig. 1）に示す．HBs抗原消失までの期間は平均1.5±2.2カ月（GTA 2.8±2.6カ月，GTB 1.0±0.6カ月，GTC 1.7±1.5カ月）であった（GTA vs GTB；$p<0.01$，GTA vs GTC；$p<0.05$）．GTAでは47例中14例（29.8%）が3カ月以上HBs抗原陽性を持続した．また3例ではHBs抗原陽性が6カ月以上持続し，そのうち2例は後にHBs抗原が消失し1例は慢性化した．また，ALT正常化までに要した期間は平均2.7±2.94カ月（GTA 3.3±4.2カ月，GTB 2.1±1.1カ月，GTC 2.2±1.1カ月）であった（GT間に有意差なし）．

発症時のALT値と臨床経過，発症時のHBe抗原の有無と臨床経過の間には一定の傾向は認められなかった．

考察：GTA HBVは男性同性間性交渉及び風俗店での性交渉により急速に拡大していることを我々は既に報告してきた[1)]．本邦におけるHBVキャリアでのGT分布に関する2回の全国調査からはGTAに感染したキャリアが増えていることも示されている[2)3)]．

GTAの症例は今回報告した通り，HBs抗原陽性の時期が長く，慢性化例の報告もあることから二次感染を起こす可能性が高い．従って治癒即ちHBs抗原の消失を確認する必要がある．ところが若年のB型急性肝炎の症例，殊にGTAに感染した症例はHBs抗原陽性のまま通院を打ち切る傾向がある．自覚症状が消失してしまうこと，慢性肝炎が最終的に肝硬変/肝細胞癌に至り得る疾患であることに対する理解が不十分であること，経済的に余裕がないことなどがその主な理由と思われる．このような例が感染を拡大させている可能性があり，他のSTD同様啓発活動が重要である．我々も通院を中断した例に対しては，書面で通院の勧告を行っているが，来院しない例が大部分である．このような通院中断例を通じた感染拡大を防止する意味でも，ユニバーサルHBワクチンの導入を真剣に検討する時期に来ていると思われる．

結論：GTAの症例ではHBs抗原陽性が他のGTに比べて長期にわたり持続する．HBVの感染拡大を防止するためにもユニバーサルHBワクチンの導入を検討すべきである．

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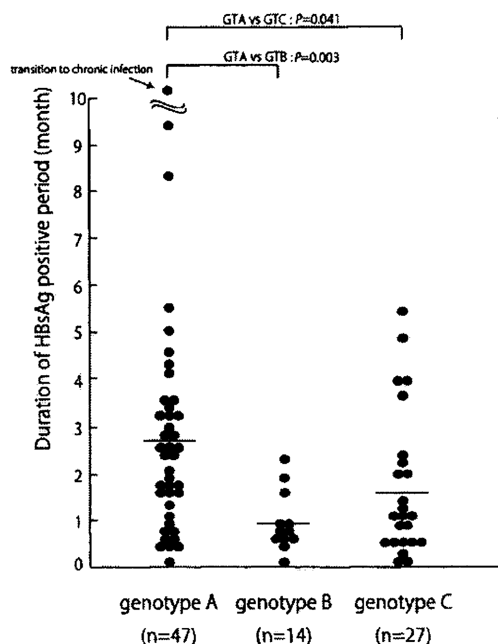


**Fig. 1** Duration of HBs antigenemia in patients with acute HBV infection. The duration is longer in patients with genotype A than those with genotype B or C.

索引用語：B 型肝炎ウイルス，遺伝子型，ユニバーサルワクチン

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## 英文要旨

### Duration of HBs antigenemia in patients with acute hepatitis B

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Recently distribution of hepatitis B virus (HBV) genotypes (GT) in the patients with acute HBV infection has been changing. It has been suggested that acute hepatitis caused by GTA HBV becomes chronic more often than that by other genotypes. We studied HBsAg-positive period in 88 patients with various HBV genotypes. HBsAg-positive period in GTA HBV is longer than that in GTB and GTC. HBsAg-positive period exceeded 6 months in 3 of 47 patients with GTA HBV. One of the three patients became chronic. GTA HBV, which is detected more than half of the patients, is related to prolonged or chronic outcome. Universal HBV vaccination program for the prevention of HBV infection should be launched in the near future.

**Key words**: hepatitis B virus, genotype, universal vaccination

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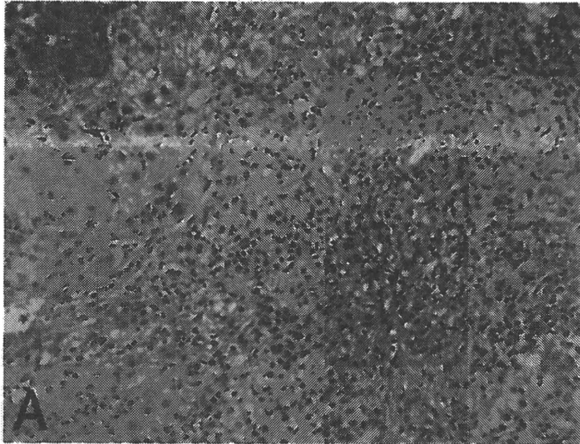
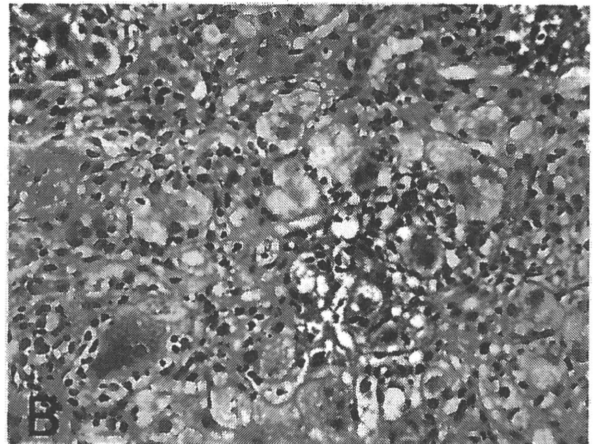
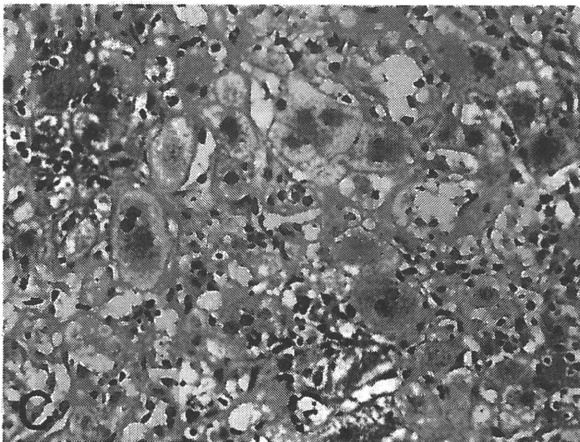
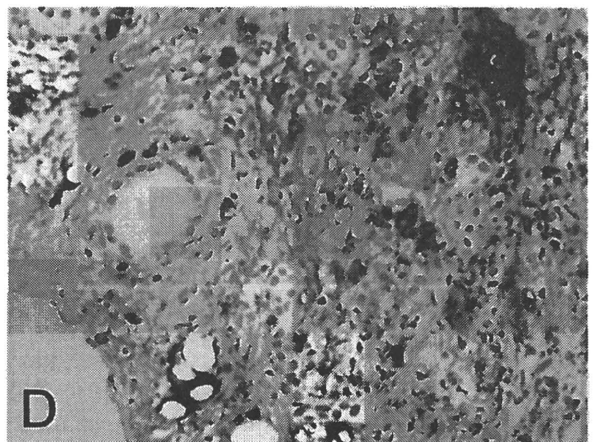
IMAGE OF THE MONTH

# Another Cause of Autoimmune Hepatitis









A 42-year-old man was admitted to our hospital because of elevated liver enzymes (aspartate aminotransferase, 642 IU/L [normal range: 12-37]; alanine aminotransferase, 788 IU/L [normal range: 7-45]; alkaline phosphatase, 605 IU/L [normal range: 124-367]; $\gamma$-glutamyl transpeptidase, 180 IU/L [normal range: 6-30]; and total bilirubin, 8.6 mg/dL [normal range: 0.3-1.2]). His serum immunoglobulin G (IgG) concentration was 5622 mg/dL (normal range: 870-1700), and anti-nuclear antibody titer (1:20480), anti-double-stranded DNA (>400 IU/mL), and smooth muscle antibody titer (1:40) were all abnormal. Infection with hepatitis A, B, and C; cytomegalovirus; and Epstein-Barr virus were excluded, and no drug use was noted. Ultrasonography, abdominal computed tomography, and magnetic resonance imaging showed no abnormalities of the extrahepatic

*Abbreviations: AIH, autoimmune hepatitis; HE, hematoxylin and eosin; IgG, immunoglobulin G.*

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bile ducts or pancreas. The first liver biopsy showed changes associated with typical autoimmune hepatitis (AIH); liver parenchyma was collapsed with broad fibrous septa containing entrapped hepatocytes, and lymphoplasmacytic infiltration with interface activity was seen (Fig. 1A; hematoxylin and eosin [H&E] staining, magnification ×200). Hepatocytes showed rosetting in numerous places (Fig. 1B; H&E staining, magnification ×400). Lobular inflammation was evident with giant cell change of hepatocytes (Fig. 1C; H&E staining, magnification ×400), but no biliary epithelial changes were found. The patient fulfilled the criteria for definite AIH by the International Autoimmune Hepatitis Group and was administered corticosteroids at 60 mg/day, which led to improvement of laboratory findings. Prior to treatment, however, the patient's serum IgG4 concentration was 642 mg/dL (normal: $\leq$ 135) in a stored serum sample, and immunostaining of liver tissue showed abundant plasma cells with strong immunohistochemical reactivity to IgG4 in a portal tract (Fig. 1D; IgG4 immunostaining, magnification ×400). A second liver biopsy performed 7 months afterward showed remaining portal sclerosis, but lobular distortion and portal inflammation were ameliorated, and serum alanine aminotransferase and IgG4 concentrations were normalized. IgG4-positive plasma cells were scarce in portal tracts (data not shown).

In an earlier report, a strong and unexpected association was seen between serum IgG4 concentration and IgG4-bearing plasma cell infiltration in the liver of a case with type 1 AIH, raising the possibility of a new disease entity termed IgG4-associated AIH.[1] Raised serum IgG4 concentration and IgG4-bearing plasma cell infiltration have a high sensitivity and specificity for the diagnosis of IgG4-related diseases.[2-4] Similar to the present case, histological findings in the liver of patients with IgG4-associated AIH showed bridging fibrosis, portal inflammation with abundant plasma cell infiltration, interface hepatitis, and lobular hepatitis. More interestingly, giant cell change and rosette formation were obvious as well. These two cases imply that IgG4-related inflammatory processes can occur in the hepatic parenchyma similarly to those in the pancreatobiliary system, and such cases may resemble AIH both clinically and pathologically. On the contrary, Chung et al. described IgG4-associated AIH as patients with AIH who had IgG4-positive plasma cells in the liver.[5] Because no cases showed high serum IgG4 in their cohort, we believe they are different from our two representative patients and thus should not be classified as an IgG4-related disease. Koyabu et al. recently reported that an IgG4/IgG1-bearing plasma cell ratio of >1 in the liver is specific for IgG4-related diseases.[6] In our patient, the IgG4/IgG1 ratio was >1 (data not shown) and consistent with their findings, which provides further evidence of our case as an IgG4-related disorder. Because IgG4-associated AIH is clearly an IgG4 hepatopathy, this disease should be differentiated from classical AIH. Detection of IgG4 and assessment of liver histology using IgG4 immunostaining may be useful for distinguishing IgG4-related diseases from classical AIH.

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Research Article

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# Association analysis of *cytotoxic T-lymphocyte antigen 4* gene polymorphisms with primary biliary cirrhosis in Japanese patients

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**Background & Aims:** Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease of still unidentified genetic etiology that is characterized by chronic inflammation of the liver. Since *cytotoxic T-lymphocyte antigen 4* (*CTLA4*) polymorphisms have recently been linked with PBC susceptibility in studies on Caucasians, we investigated the genetic association between *CTLA4* polymorphisms and PBC in a Japanese population.
**Methods:** Five single nucleotide polymorphisms (SNPs) in the *CTLA4* gene (rs733618, rs5742909, rs231775, rs3087243, and rs231725) were genotyped in 308 patients with PBC and 268 healthy controls using a TaqMan assay.
**Results:** One *CTLA4* gene SNP (rs231725) was significantly associated with susceptibility to anti-mitochondrial antibody (AMA)-positive PBC, but clinical significance disappeared after correction for multiple testing. Moreover, *CTLA4* gene SNPs did not influence AMA development or disease progression to orthotopic liver transplantation in our Japanese cohort. In haplotype analyses, one haplotype [haplotype 1 (CGGA)] at rs5742909, rs231775, rs3087243, and rs231725, was significantly associated with susceptibility to both AMA-positive PBC and overall PBC.
**Conclusions:** This study showed that *CTLA4* gene polymorphisms had a modest, but significant association with susceptibility to PBC in the Japanese population. The connection between genetic variants and the function of the *CTLA4* gene remains to be addressed in future investigations.
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Keywords: Primary biliary cirrhosis; Single nucleotide polymorphisms; Cytotoxic T-lymphocyte antigen 4; Genetic susceptibility.
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*Abbreviations:* PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibody; CTLA4, cytotoxic T-lymphocyte antigen 4; OLT, orthotopic liver transplantation; SNPs, single nucleotide polymorphisms; UTR, untranslated region; LD, linkage disequilibrium; HWE, Hardy-Weinberg equilibrium; *pc*, corrected *p*; OR, odds ratio; CI, confidence interval; sCTLA4, soluble isoform of CTLA4.

## Introduction

Primary biliary cirrhosis (PBC) is a liver-specific autoimmune disease characterized by female preponderance and the destruction of intrahepatic bile ducts that often results in cirrhosis and hepatic failure [1]. The etiology of PBC has yet to be conclusively elucidated, although genetic factors are considered to play a prominent role in family and population studies [2–5]. Prior reports have shown the HLA-*DRB1*08* allele to be a weak and regional determinant of PBC susceptibility [6–8]. However, HLA alone does not explain the entire genetic predisposition to PBC, mainly because at least 80–90% of patients with the disease do not carry the most common HLA susceptibility alleles. In this regard, other non-HLA genes are thus being considered to contribute to disease development [9,10].

PBC displays immunologically characteristic features like biliary lymphocytic infiltrates, anti-mitochondrial antibodies (AMA) against the inner lipoyl domain of the E2 subunits of the pyruvate dehydrogenase complex, and elevated serum levels of IFN-$\gamma$ and TNF-$\alpha$. The serologic hallmark of PBC is the presence of AMA [11,12], which are found in 95% of patients with PBC [13] and have a specificity of 98% for the disease [12]. Auto-reactive $CD4^+$ and $CD8^+$ T cells are also found in high concentrations in the portal triads of patients with PBC, often surrounding and infiltrating necrotic bile ducts [14–16]. A recent study suggested that a reduction in the number of $CD4^+CD25^+$ regulatory T cells in livers affected with PBC contributed to disease progression [17]. Accumulating data such as these, support a direct role of T-lymphocytes in the pathogenesis of PBC.

The cytotoxic T-lymphocyte antigen 4 (CTLA4) is an inhibitory receptor expressed on the cell surface of activated memory T cells and $CD4^+CD25^+$ regulatory T cells that acts largely as a negative regulator of T-cell responses. Since the potential inhibitory functions of CTLA4 [18] may also trigger a breakdown of immunological self-tolerance, polymorphisms affecting these processes could have significant effects on susceptibility to autoimmunity.

The *CTLA4* gene is a primary candidate for genetic susceptibility to autoimmune diseases, including type 1 diabetes, auto-

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immune hepatitis [19,20], and autoimmune pancreatitis [21]. In particular, two single nucleotide polymorphisms (SNPs), rs231775 (49AG) and rs3087243 (CT60), have been widely studied in PBC [22–24]. Although early studies found an association between SNP 49G coding and PBC [22–24], ensuing reports showed negative relationships with susceptibility [25–30] or a positive association with liver damage [31]. A recent investigation reported that rs231725 in the 3′ flanking region of *CTLA4* is associated with AMA-positive PBC in Caucasians [27]. In addition to *CTLA4* polymorphisms, HLA class II, IL12A, IL12RB, and several other candidate SNPs were disclosed as predisposition genes for PBC by a high-density genome-wide association study [9]. Since these SNPs have not been extensively examined in a large Japanese population, the present study sought to evaluate the involvement of *CTLA4* SNPs and haplotype SNPs in susceptibility to PBC and disease progression in Japanese patients.

## Patients and methods

*Subjects*

We analyzed a total of 576 subjects (308 PBC patients and 268 healthy controls) collected from two different regions of Japan (Table 1). Cohort 1 consisted of 198 patients clinically diagnosed with PBC (173 women, median age 58 years old) and 170 healthy subjects who were seen at Shinshu University Hospital, Matsumoto, Japan. Cohort 2 consisted of 110 patients clinically diagnosed with PBC (92 women, median age 61 years old) and 98 healthy subjects from the National Hospital Organization Nagasaki Medical Center, Omura, Japan. The racial background of all subjects was Japanese. Control subjects were volunteers from hospital staff who had indicated the absence of any major illnesses in a standard questionnaire. The diagnosis of PBC was based on criteria from the American Association for the Study of Liver Diseases [32]. Serum AMA, specific for the pyruvate dehydrogenase complex-E2 component, was measured by the enzyme-linked immunosorbent assay as reported previously [33]. An index of greater than seven was considered a positive result. All patients were negative for hepatitis B surface antigen, antibody to hepatitis C virus, and antibody to human immunodeficiency virus. To evaluate associations between SNPs and disease progression, patients were classified into two stages based on their most recent follow-up [34]: early stage patients were histologically in Scheuer stage I or II [35,36] or of unknown histological stage without liver cirrhosis, and late stage patients were histologically in Scheuer stage III or IV or clinically diagnosed with liver cirrhosis or hepatic failure. All participants provided informed written consent for this study, which had been approved by the institutional ethics committee.

*CTLA4 SNP genotyping*

Genomic DNA from patients and controls was isolated by phenolic extraction of sodium dodecyl sulfate-lysed and proteinase K-treated cells, as described previously [37,38], and adjusted to 10–15 ng/μl.

The five *CTLA4* gene SNPs examined in this study (rs733618, rs5742909, rs231775, rs3087243, and rs231725) were genotyped using the 5′ nuclease (TaqMan) assay using primer, probes, and reaction conditions as recommended by the manufacturer (Applied Biosystems, Tokyo, Japan). These SNPs were selected based on previous reports [21–23,26,27], and were all located in the *CTLA4* gene; SNPs rs733618 and rs5742909 were in the promoter region, SNP rs231775 in exon 1, and SNPs rs3087243 and rs231725 in the 3′ untranslated region (UTR). Polymerase chain reaction was performed with a TaqMan Assay for Real-Time PCR (7500 Real-Time PCR System; Applied Biosystems) following the manufacturer's instructions.

*Haplotype-genotype estimation*

The R package "haploview" [39] was used to evaluate the haplotype structure of the five examined *CTLA4* SNPs. Pairwise linkage disequilibrium (LD) patterns and haplotype frequency analysis for all SNPs in patients and controls were assessed by the block definition by Gabriel et al. [40].

**Table 1. Demographic and clinical data of patients with PBC at study onset.**

| Characteristics | Cohort 1 Shinshu $n = 198$ | Cohort 2 Nagasaki $n = 110$ | Combined $n = 308$ |
|---|---|---|---|
| Age, years[a] | 58 (30–83) | 61 (34–85) | 58 (30–88) |
| Female/Male | 173/25 | 92/18 | 265/43 |
| Disease progression | | | |
| Early stage, *n*/Late stage, *n* | 149/49 | 74/36 | 223/85 |
| Orthotopic liver transplantation, *n* (%) | 15 (7.6) | 2 (1.8) | 17 (5.5) |
| AMA positive, *n* (%) | 171 (86.4) | 102 (92.8) | 273 (88.6) |

PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibody specific for the pyruvate dehydrogenase complex-E2 component.
[a] Median (range).

*Statistical analysis*

The Hardy–Weinberg equilibrium (HWE) test was done for each SNP between control and patient groups. The significance of allele distribution between PBC patients and healthy controls was assessed using the $\chi^2$-test with the use $2 \times 2$ or $2 \times 3$ comparisons. Fisher's exact probability test was used for groups with fewer than 5 samples. A *p* value of less than 0.05 was considered statistically significant; *p* values were corrected using Bonferroni's correction by multiplying by the number of different alleles observed in each locus (*pc*).

## Results

In total, five SNPs located in the *CTLA4* gene were genotyped in 198 patients with PBC and 170 healthy controls in cohort 1 and 110 patients with PBC and 98 healthy controls in cohort 2 (Table 2). Hardy-Weinberg equilibrium (HWE) was observed for all 5 of the examined SNPs in both control groups, and the minor allele frequencies of all SNPs were greater than 5%. In cohort 1, one SNP (rs733618) differed significantly from HWE ($p = 0.03$) (Table 2), and the frequency of the minor A allele at rs231775 was significantly decreased (33.9% vs. 41.5%, odds ratio (OR) 0.72, 95% confidence interval (95% CI) 0.53–0.99, $p = 0.042$, $pc = 0.209$) in 171 AMA-positive PBC patients compared with controls. Positivity for the major G allele (A/G + G/G) at rs231775 was significantly higher in patients with AMA-positive PBC than in healthy subjects (88.3% vs. 79.1%, OR 1.96, 95% CI 1.08–3.53, $p = 0.026$, $pc = 0.128$). Additionally, the allele frequency (61.7% vs. 53.2%, OR 1.41, 95% CI 1.04–1.92, $p = 0.025$, $pc = 0.127$) and allele carrier frequency (86.0% vs. 75.9%, OR 1.96, 95% CI 1.12–3.41, $p = 0.018$, $pc = 0.089$) of the major A allele at rs231725 were significantly increased in AMA-positive PBC patients compared with healthy controls. However, these statistical significances disappeared after correction for multiple testing. No significant differences were observed among the 5 SNPs in cohort 2. The allele frequency (60.3% vs. 53.4%, OR 1.33, 95% CI 1.04–1.69, $p = 0.022$) of the major A allele at rs231725 was significantly increased in combined analysis (cohorts 1 and 2) of 273 AMA-positive PBC patients compared with 268 healthy controls (Table 3), but statistical significance was lost after correction for multiple testing ($pc = 0.110$) (Table 3).

Pairwise LD mapping confirmed that all alleles were in strong LD with an index of >0.8. A strong LD was detected in the same block for PBC patients and controls. We next evaluated haplotype association among AMA-positive PBC patients and healthy subjects in a combined analysis. To estimate haplotype frequencies and analyze haplotype association with PBC, we selected tag SNPs

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**Table 2. Allele frequencies of SNPs in the *CTLA4* gene in PBC patients and controls.**

| SNP No. | dbSNP | Allele major/minor | Position (bp) | Gene location | Cohort 1 (Shinshu) | | | | Cohort 2 (Nagasaki) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Patients ($n$ = 198) | | Controls ($n$ = 170) | | Patients ($n$ = 110) | | Controls ($n$ = 98) | |
| | | | | | MAF (%) | HWE $p$ value | MAF (%) | HWE $p$ value | MAF (%) | HWE $p$ value | MAF (%) | HWE $p$ value |
| 1 | rs733618 | T/C | 204439189 | Promoter | 44.4 | 0.030 | 39.1 | 0.071 | 39.5 | 0.570 | 43.4 | 0.366 |
| 2 | rs5742909 | C/T | 204440592 | Promoter | 9.1 | 0.347 | 11.2 | 0.295 | 13.2 | 0.828 | 13.8 | 0.514 |
| 3 | rs231775 | G/A | 204440959 | Exon 1 | 35.4 | 0.784 | 41.5 | 0.089 | 39.5 | 0.334 | 41.8 | 0.827 |
| 4 | rs3087243 | G/A | 204447164 | 3′ UTR | 26.3 | 0.994 | 30.3 | 0.709 | 26.4 | 0.125 | 31.1 | 0.316 |
| 5 | rs231725 | A/G | 204448920 | 3′ UTR | 39.9 | 1.000 | 46.8 | 0.288 | 41.8 | 0.586 | 46.4 | 1.000 |

MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; UTR, untranslated region.

**Table 3. Allele frequencies of 5 SNPs in 273 AMA⁺ patients with PBC and 268 healthy subjects.**

| SNP No. | Allele | Patients* | Controls* | $p$ | $pc$ | OR | 95% CI |
|---|---|---|---|---|---|---|---|
| 1 | C | 43.2 | 40.7 | 0.395 | 1.975 | 1.11 | 0.87–1.41 |
| | T | 56.8 | 59.3 | | | | |
| 2 | C | 89.6 | 87.9 | 0.380 | 1.900 | 1.18 | 0.81–1.73 |
| | T | 10.4 | 12.1 | | | | |
| 3 | G | 63.9 | 58.4 | 0.062 | 0.310 | 1.26 | 0.99–1.61 |
| | A | 36.1 | 41.6 | | | | |
| 4 | G | 74.4 | 69.4 | 0.070 | 0.350 | 1.28 | 0.98–1.67 |
| | A | 25.6 | 30.6 | | | | |
| 5 | A | 60.3 | 53.4 | 0.022 | 0.110 | 1.33 | 1.04–1.69 |
| | G | 39.7 | 56.6 | | | | |

AMA, anti-mitochondrial antibodies; PBC, primary biliary cirrhosis; OR, odds ratio; $pc$, corrected $p$ value; 95% CI, 95% confidence interval; *, frequency (%).
$p$ value was calculated by a $\chi^2$-test 2 × 2 contingency table (df = 1).

**Table 4. *CTLA4* haplotypes in 273 AMA⁺ patients with PBC and 268 healthy subjects.**

| Haplotype | SNP No. 2 | 3 | 4 | 5 | Patients* ($n$ = 546) | Controls* ($n$ = 536) | $p$ | OR | 95% CI |
|---|---|---|---|---|---|---|---|---|---|
| 1 | C | G | G | A | 59.7 | 51.9 | 0.0095 | 1.37 | 1.08–1.75 |
| 2 | C | A | A | G | 25.5 | 29.4 | 0.1464 | 0.82 | 0.62–1.07 |
| 3 | T | A | G | G | 10.3 | 11.8 | 0.4186 | 0.85 | 0.58–1.25 |
| 4 | C | G | G | G | 3.8 | 5.4 | 0.2153 | 0.70 | 0.39–1.23 |

PBC, primary biliary cirrhosis; OR, odds ratio; 95% CI, 95% confidence interval; *, proportion of indicated haplotype (%).
Values for $n$ indicate two times the number of individuals since each person carries two haplotypes.
$p$ value was calculated by a $\chi^2$-test 2 × 2 contingency table (df = 1).

using the Tagger algorithm from the Haploview program. Four tag SNPs (SNPs 2–5: rs5742909, rs231775, rs3087243, and rs231725) were selected to capture most of the allelic diversity in the two cohorts. The four estimated haplotypes showed a frequency of >5% in 11 haplotypes created by expectation–maximization algorithms (Table 4). Haplotype 1 (CGGA) was significantly associated with AMA-positive PBC susceptibility (59.7% vs. 51.9%, OR 1.37, 95% CI 1.08–1.75, $p$ = 0.0095). No other haplotypes were associated with either susceptibility or resistance to PBC.

Evaluation of the 5 *CTLA4* SNPs between AMA-positive and AMA-negative subgroups revealed neither significant allelic associations (Table 5) nor significant haplotype associations (Table 6), even when compared with early or late stages (Tables 5 and 6). Moreover, a comparison of 17 orthotopic liver transplantation (OLT) PBC cases and 291 non-OLT cases revealed no significant differences in allele frequencies (Table 5). In haplotype analysis, no statistical associations were found with OLT (Table 6).

## Discussion

This study revealed that haplotype 1 (CGGA) was significantly associated with disease susceptibility in 273 AMA-positive PBC patients, as well as overall in all 308 PBC patients ($p$ = 0.012) (data not shown). This finding is in agreement with the Caucasian study by Juran et al. [27], and thus constitutes a promising susceptibility gene candidate. However, since the precise function of *CTLA4* SNPs remains undefined, we cannot exclude the possibility that these SNPs may only be a linkage marker for a yet unidentified SNP within the *CTLA4* gene. Sequencing of the entire gene and assessing the functional role of these SNPs will be required.

SNP rs231775 associated with PBC is commonly referred to as 49AG in several studies [23,24,27,31,41]. Our finding corroborated a previous report [31], in which 49AG was not associated with susceptibility to PBC but there was a discrepancy in associ-

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**Table 5. Allele frequencies of *CTLA4* SNPs in AMA, histological or clinical disease progression, and OLT states.**

| SNP No. | Allele | AMA⁺* (n = 273) | AMA⁻* (n = 35) | p | Early* (n = 223) | Late* (n = 85) | p | Non-OLT* (n = 291) | OLT* (n = 17) | p |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | C | 43.2 | 38.6 | 0.459 | 44.4 | 38.2 | 0.167 | 42.6 | 44.1 | 0.863 |
| | T | 56.8 | 61.4 | | 55.6 | 61.8 | | 57.4 | 55.9 | |
| 2 | C | 89.6 | 88.6 | 0.800 | 90.0 | 89.2 | 0.783 | 89.3 | 91.2 | 0.736 |
| | T | 10.4 | 11.4 | | 10.0 | 10.8 | | 10.7 | 8.8 | |
| 3 | G | 63.9 | 57.1 | 0.267 | 63.9 | 61.2 | 0.531 | 62.9 | 67.6 | 0.576 |
| | A | 36.1 | 42.9 | | 36.1 | 38.8 | | 37.1 | 32.4 | |
| 4 | G | 74.4 | 68.6 | 0.300 | 74.7 | 71.2 | 0.380 | 73.7 | 73.5 | 0.981 |
| | A | 25.6 | 31.4 | | 25.3 | 28.8 | | 26.3 | 26.5 | |
| 5 | A | 60.3 | 52.9 | 0.235 | 60.8 | 55.9 | 0.270 | 59.5 | 58.8 | 0.942 |
| | G | 39.7 | 47.1 | | 39.2 | 44.1 | | 40.5 | 41.2 | |

PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibodies; OLT, orthotopic liver transplantation; SNP, single nucleotide polymorphism; *, frequency (%).
p value was calculated by a $\chi^2$-test 2 × 2 contingency table (df = 1).

**Table 6. Comparison of *CTLA4* haplotype frequencies in AMA, histological or clinical disease progression, and OLT states.**

| Haplotype | SNPs No. 2 | 3 | 4 | 5 | AMA⁺* (n = 546) | AMA⁻* (n = 70) | p | Early* (n = 446) | Late* (n = 170) | p | Non-OLT* (n = 582) | OLT* (n = 34) | p |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | C | G | G | A | 60.1 | 52.8 | 0.245 | 60.5 | 55.9 | 0.292 | 59.3 | 58.8 | 0.959 |
| 2 | C | A | A | G | 25.5 | 30.0 | 0.415 | 25.1 | 28.2 | 0.430 | 26.1 | 23.5 | 0.738 |
| 3 | T | A | G | G | 10.3 | 10.0 | 0.947 | 10.3 | 10.0 | 0.909 | 10.3 | 8.8 | 0.781 |
| 4 | C | G | G | G | 3.5 | 4.3 | 0.720 | 3.1 | 4.7 | 0.346 | 3.4 | 5.9 | 0.458 |

PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibodies; OLT, orthotopic liver transplantation; SNP, single nucleotide polymorphism; *, proportion of indicated haplotype (%).
Values for n indicate two times the number of individuals since each person carries two haplotypes. p value was calculated by a $\chi^2$-test 2 × 2 contingency table (df = 1).

ation with liver damage that might have arisen from the number of cases analyzed. 49AG also appears to affect cell surface expression of CTLA4 by CTLA4-driven down-regulation in response to T-cell activation [42]. This coding polymorphism is located in a signal peptide that is cleaved from the functional protein, and has been shown to affect glycosylation of the autoimmune susceptibility G allele, resulting in diminished processing efficiency and thus decreased trafficking to the cell surface [43]. It will be necessary to confirm the functional difference between patients with these SNPs and T-cell activation in a future study.

The rs3087243 SNP, also referred to as CT60, is located in the 3′ UTR of the *CTLA4* gene and reported to influence the production of the soluble isoform of CTLA4 (sCTLA4). The sCTLA4 mRNA encoded by the +CT60G-allele is produced at a reduced rate compared with that encoded by the A allele. As sCTLA4, which is secreted by resting T cells, is a suppressor of T-cell activation, it is conceivable that carriers of the +CT60G-allele allele may be more susceptible to autoimmune diseases [44]. Although studies from Canada and Italy found an association between PBC and the CT60 SNP [29,41], other studies have since failed to confirm this association [27,28], including ours.

In haplotype analysis, haplotype 1 contained all of the known SNP risk alleles that have been functionally determined in other disease studies. These include the C allele at −318, which has been found to affect the expression of CTLA4 mRNA cell surface expression [45], the minor G allele at 49AG, reported to reduce cell surface expression of CTLA4 [42], and the G allele of CT60, which affects the expression of the soluble form of the CTLA4 molecule, indicating the possibility that this haplotype might contribute to PBC susceptibility in the Japanese population.

Lastly, Juran et al. have suggested that CTLA4 plays a role in influencing AMA development as well as progression to OLT in PBC based on their haplotype analyses [27]. Our data revealed no statistical significance in regards to AMA development or disease progression to cirrhosis or OLT, possibly due to the number of patients showing AMA negativity and proceeding to OLT being too small to evaluate. Another consideration is that disease progression in Japanese patients might have a stronger association with positivity for anti-gp210 antibodies as a risk factor of progression to hepatic failure than *CTLA4* polymorphisms [46]. Further longitudinal follow-up studies in larger cohorts are required to resolve this critical question.

In conclusion, we found that *CTLA4* gene polymorphisms had a modest, but significant, association with susceptibility to PBC in the Japanese population and may share a common susceptibility haplotype with Caucasians. The connection between genetic variants and the function of the *CTLA4* gene remains to be addressed in future investigations.

#### Conflict of Interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Journal of Medical Virology 82:539–545 (2010)

# Incidence of Hepatocellular Carcinoma in Patients With Chronic Hepatitis B Virus Infection Who Have Normal Alanine Aminotransferase Values

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The importance of alanine aminotransferase (ALT) levels in the progression of hepatitis B virus (HBV) infection remains a subject of debate. This study sought to identify independent risk factors involved in development of hepatocellular carcinoma (HCC), particularly in patients with chronic HBV infection who have normal ALT values. Data from 381 consecutive hepatitis B patients were analyzed with average ALT integration values ≤40 IU/L and follow-up periods of >3 years. Integration values were calculated from biochemical tests, and serological markers associated with the cumulative incidence of HCC were analyzed. HCC developed in 17 of the 381 patients (4.5%) during the follow-up period. Male sex (hazard ratio, 6.011 [95% confidence interval: 1.353–26.710], $P = 0.018$), high HBV-DNA levels (≥5.0 log copies/ml; 5.125 [1.880–13.973], $P = 0.001$), low platelet counts ($<15.0 \times 10^4/mm^3$; 4.803 [1.690–13.647], $P = 0.003$), and low total cholesterol levels (<130 mg/dl; 5.983 [1.558–22.979], $P = 0.009$) were significantly associated with greater incidence of HCC development. High HBV-DNA levels and low platelet counts are associated with the development of HCC in patients infected with hepatitis B who have normal ALT values. Therefore, maintenance of low HBV-DNA levels is important for the prevention of HCC in patients with low platelet counts, particularly in patients whose ALT values fall within the current normal range. ***J. Med. Virol. 82:539–545, 2010.*** © 2010 Wiley-Liss, Inc.

**KEY WORDS:** hepatitis B virus (HBV); HBV-DNA; normal alanine aminotransferase; platelet counts; hepatocellular carcinoma

## INTRODUCTION

Worldwide, an estimated 350 million individuals are infected chronically with hepatitis B virus (HBV), and 1 million die each year from HBV-related liver disease [EASL Jury, 2003]. Chronic HBV infection is a major risk factor for the development of hepatocellular carcinoma (HCC) [Beasley, 1988; EASL Jury, 2003]. Patients who test positive for the hepatitis B surface antigen (HBsAg) have a 70-fold greater risk of developing HCC compared with HBsAg-negative patients [Szmuness, 1978; Beasley et al., 1981]. HBV infection is endemic in Southeast Asia, China, Taiwan, Korea, and sub-Saharan Africa, where up to 85–95% of patients with HCC are HBsAg-positive [Rustgi, 1987]. HCC is the third and fifth leading cause of death from malignant neoplasms in Japanese men and women, respectively, and the death rate from HCC has increased markedly in Japan since 1975 [Kiyosawa et al., 2004]. Hepatitis C virus (HCV)-related HCC accounts for 75% of all cases of HCC in Japan, while HBV-related HCC accounts for 15% of such cases [Kiyosawa et al., 2004].

Although an increasing body of epidemiological and molecular evidence suggests that HBV is associated with the development of HCC, the exact role of HBV in carcinogenesis is unclear [Ikeda et al., 2005; Wong et al., 2006]. HBV elicits a chronic necroinflammatory hepatic disease [Yu and Chen, 1994], and liver injury associated with HBV infection is mediated by viral factors in addition to the host immune response. Patients who are positive for the hepatitis B e antigen (HBeAg) commonly have increased hepatic inflammatory activity and an increased risk of developing HCC [Yang et al., 2002]. HBeAg-negative HBsAg carriers who retain high levels of HBV-DNA and show persistent necroinflammation of the liver have an increased risk of acquiring HCC [Yu et al., 2005; Chen et al., 2006].

The authors report no conflicts of interest.

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Alanine aminotransferase (ALT) activity is the most widely used laboratory test for the evaluation of necroinflammatory activity in liver disease [Prati et al., 2002]; however, it is well known that HCC occurs in some HBsAg carriers with normal ALT values. Recently, Chen et al. [2006] conducted a large cohort study in Taiwan and found that elevated serum HBV-DNA levels are strong predictive factors for the development of HCC, independent of the ALT values. It is an important problem for early detection of HCC that general practitioners are sometimes unaware of those patients with normal ALT as high-risk subjects for HCC. There is little information about how many patients with normal ALT develop HCC. It is important that ALT values should be expressed with integration values to ensure a valid analysis, since ALT values fluctuate frequently [Kumada et al., 2007]. Therefore, this study sought to identify the independent risk factors, involving mainly serological markers, associated with the development of HCC in patients infected chronically with HBV with average ALT integration values ≤40 IU/L.

## MATERIALS AND METHODS

### Patient Selection

A total of 1,861 consecutive patients who were positive for HBsAg visited the Department of Gastroenterology at Ogaki Municipal Hospital, Japan, between September 1994 and August 2003. After assessing each patient's long-term prognosis, 381 consecutive patients were selected for further study who (1) were positive for HBsAg for at least 6 months; (2) displayed no evidence of HCV infection; (3) had no other possible causes of chronic liver disease (i.e., alcohol consumption lower than 80 g/day, no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) had a follow-up period of >3 years; (5) had no evidence of HCC for at least 3 years from the start of the follow-up period; (6) had no history of therapy involving interferons, nucleosides, or nucleotide analogues; (7) had ALT measurements taken more than twice in a year; and (8) had average ALT integration values ≤40 IU/L (Fig. 1).

Patients were evaluated at the hospital at least every 6 months. During each follow-up examination, platelets, ALT, aspartate aminotransferase (AST), gamma glutamyl transpeptidase (gamma-GTP), total bilirubin, cholinesterase, alkaline phosphatase (ALP), albumin, total cholesterol, HBeAg, anti-HBe, HBV-DNA, and alpha-fetoprotein (AFP) were measured at least every 6 months. Commercial radioimmunoassay kits were used to test blood samples for HBsAg, HBeAg, and anti-HBe (Abbott Japan Co., Ltd, Tokyo, Japan). Before July 2001, serum HBV-DNA concentrations were monitored using the amplification-hybridization protection assay (DNA probe, Chugai-HBV; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) with a lower detection limit of ~5,000 viral genome copies/ml (3.7 log copies/ml). After August 2001, serum HBV-DNA levels were monitored using the polymerase chain reaction (PCR) (COBAS Amplicor HBV monitor test, Roche Diagnostics K.K., Tokyo, Japan) with a lower detection limit of ~400 viral genome copies/ml (2.6 log copies/ml). HBV genotyping was carried out as described previously [Kato et al., 2001]. ALT, AST, gamma-GTP, ALP, and AFP were expressed as integration values [Kumada et al., 2007]. When ALT was used as an example, the integration value of ALT was calculated as follows: $(y_0+y_1)\times x_1/2+(y_1+y_2)\times x_2/2+(y_2+y_3)\times x_3/2+(y_3+y_4)\times x_4/2+(y_4+y_5)\times x_5/2+(y_5+y_6)\times x_6/2+(y_6+y_7)\times x_7/2+(y_7+y_8)\times x_8/2$ (Fig. 2). The area of a trapezoid with ALT value was calculated and the measurement interval and added the values. The

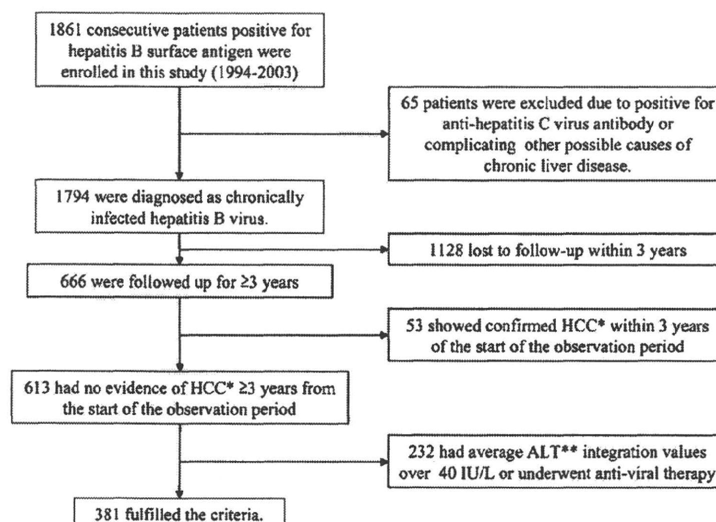


Fig. 1. Schematic flowchart of enrolled patients. *, hepatocellular carcinoma (HCC); **, alanine aminotransferase (ALT).

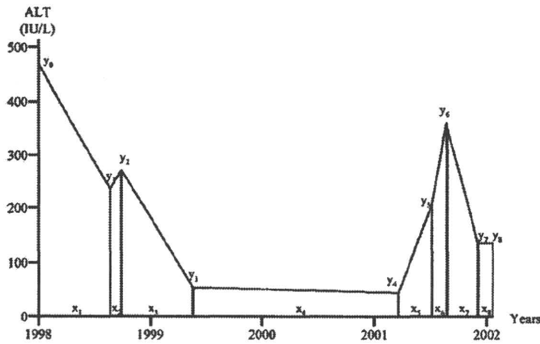


Fig. 2. Integration value of alanine aminotransferase (ALT). The integration value of ALT was calculated as follows: $(y_0 + y_1) \times x_1/2 + (y_1 + y_2) \times x_2/2 + (y_2 + y_3) \times x_3/2 + (y_3 + y_4) \times x_4/2 + (y_4 + y_5) \times x_5/2 + (y_5 + y_6) \times x_6/2 + (y_6 + y_7) \times x_7/2 + (y_7 + y_8) \times x_8/2$. The integration value of ALT was divided by the observation period and expressed as an average integration value.

integration value of ALT was divided by the observation period to obtain the average integration value (Fig. 3). In addition, patients were classified into two groups according to the change of pattern of ALT: persistently normal ALT group and intermittently normal ALT group. The persistently normal ALT group included patients with persistently normal ALT values ≤40 IU/L during follow-up period. The intermittently normal ALT group included patients with temporary ALT fluctuations but the average integration value was ≤40 IU/L. Platelet counts, total bilirubin, cholinesterase, albumin, total cholesterol, HBeAg, anti-HBe, and HBV-DNA were analyzed at the time of entry into the study.

Ultrasonograpy was performed in all patients at the start of the follow-up period for the evaluation of liver fibrosis. The diagnosis of cirrhosis was made according to typical ultrasound findings, for example, superficial nodularity, a coarse parenchymal echo pattern, and

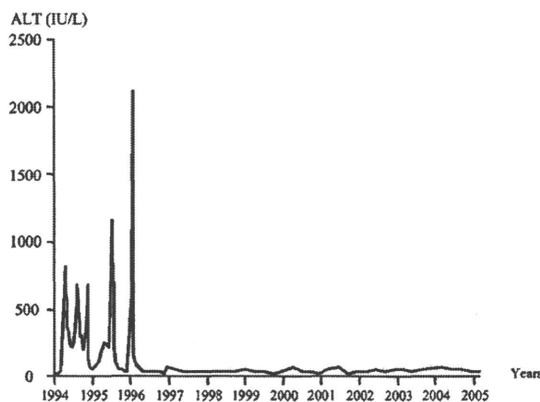


Fig. 3. Average integration value and arithmetic mean value of alanine aminotransferase (ALT) in a 26-year-old patient with hepatitis B virus (HBV). The patient was followed-up for 11.2 years. The number of ALT examinations was 96. The integration value of ALT was 955.2 IU/L × years. The average integration value was 85.3 IU/L, whereas the arithmetic mean value was 255.6 IU/L. This difference is due to the number of ALT measurements between a period of high ALT level and low ALT level.

signs of portal hypertension (splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [Caturelli et al., 2003; Iacobellis et al., 2005; Shen et al., 2006].

To detect early-stage HCC, ultrasonography, computed tomography, magnetic resonance imaging, and/or measurement of tumor markers (i.e., AFP, *Lens culinaris* agglutinin-reactive AFP, and des-γ-carboxyprothrombin) were performed for all patients, at least every 6 months. Blood biochemistry data used in this study were obtained over 1 year prior to HCC development. The study ended in December 31, 2007 or on the date of HCC identification, whichever was earlier. The diagnosis of HCC was based on histological examination (n = 9). In the remaining eight patients, the diagnosis was based on clinical criteria [Kudo, 1999; Torzilli et al., 1999].

### Statistical Analysis

Statistical analyses were performed using the Statistical Program for Social Science (SPSS version 17.0 for Windows; SPSS Japan, Inc., Tokyo, Japan). Continuous variables are expressed as median (range). The Kruskal–Wallis test was used to assess continuous variables with a skewed distribution, and the chi-square test was used to assess categorical variables. An actuarial analysis of the cumulative incidence of HCC was performed using the Kaplan–Meier method, and differences were tested by a log-rank test. The Cox proportional hazard model and forward selection method were used to estimate the relative risk of HCC development associated with age (i.e., ≤40 years or >40 years), sex (i.e., male or female), HBeAg (i.e., positive or negative), HBV-DNA level (i.e., <5.0 or ≥5.0 log copies/ml), average ALT integration value (i.e., ≤20 or >20 IU/L), the change pattern of ALT (persistently normal ALT group or intermittently normal ALT group), average AST integration value (i.e., ≤40 or >40 IU/L), platelet count (i.e., <15.0 or ≥15.0 × $10^4$/mm$^3$), average gamma-GTP integration value (i.e., ≤56 or >56 IU/L), total bilirubin (i.e., ≤1.2 or >1.2 mg/dl), average ALP integration value (i.e., ≤338 or >338 IU/L), cholinesterase (i.e., <431 or ≥431 IU/L), albumin (i.e., <3.5 or ≥3.5 g/dl), total cholesterol (i.e., <130 or ≥130 mg/dl), and average AFP integration value (i.e., ≤10 or >10 ng/ml). The lower and upper limits of the reference values at our institution were used as cut-off values for AST, platelet count, gamma-GTP, total bilirubin, ALP, cholinesterase, albumin, and total cholesterol. Statistical significance was defined as $P < 0.05$.

The study protocol was approved by the Ethics Committee at Ogaki Municipal Hospital and performed in compliance with the Helsinki Declaration.

## RESULTS

### Patient Characteristics

The median follow-up period was 8.6 years (range, 3.0–14.0 years). HCC developed in 17 of 381 patients

(4.5%) during the follow-up period. The 5- and 10-year cumulative incidence of HCC was 0.8% and 6.5%, respectively. Profiles and data from the 381 patients with normal ALT values are summarized in Table I.

### Factors Associated With the Incidence of HCC

Factors associated with the incidence of HCC, as determined by univariate analysis, are listed in Table II. Male sex, high HBV-DNA levels, intermittently normal ALT, high AST levels, low platelet counts, low cholinesterase levels, low albumin levels, low total cholesterol levels, high AFP levels, and presence of cirrhosis were significantly associated with HCC development. The cumulative incidence of HCC was significantly higher in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70) than in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311, $P < 0.001$, Fig. 4). The cumulative incidence of HCC was significantly higher in patients with HBV-DNA levels $\geq$5.0 log copies/ml (n = 90) than in patients with HBV-DNA levels <5.0 log copies/ml (n = 291, $P < 0.001$, Fig. 5).

Factors associated with incidence of HCC, as determined by the Cox proportional hazard model and the forward selection method, are listed in Table III. Male sex, high HBV-DNA levels, low platelet counts, and low total cholesterol levels were significantly associated with the development of HCC.

Baseline of patients with normal ALT according to HBV-DNA level and platelet counts.

HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts $<15.0 \times 10^4/mm^3$ [n = 45]; C: HBV-DNA levels $\geq$5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 54]; and D: HBV-DNA levels $\geq$5.0 log copies/ml and platelet counts $<15.0 \times 10^4/mm^3$ [n = 25]). Positive rates of HBeAg were highest in Group C, total cholesterol levels were lowest in Group D, and ALT level, frequency of intermittently normal ALT, AFP levels, and presence

TABLE I. Patient Characteristics

| | |
|---|---|
| Age (years) | 49 (12–84) |
| Sex (F/M) | 201/180 |
| BMI (kg/m$^2$) | 22.4 (17–36) |
| HBV genotype (A/B/C/D) | 8/24/149/2 |
| HBeAg (positive/negative) | 59/322 |
| HBV-DNA (log copies/ml) | 3.7 (2.6–9.6) |
| ALT (IU/L) | 22.6 (8.7–39.9) |
| Persistently normal ALT (+/−)[a] | 182/199 |
| AST (IU/L) | 23.4 (13.3–74.3) |
| Platelet ($\times 10^4$/mm$^3$) | 19.3 (3.3–39.5) |
| Gamma-GTP (IU/L) | 19.5 (7.4–441.0) |
| Total bilirubin (mg/dl) | 0.6 (0.3–4.7) |
| ALP (IU/L) | 214.8 (82.4–621.3) |
| Cholinesterase (IU/L) | 314.0 (99.6–483.9) |
| Albumin (g/dl) | 4.2 (2.4–4.9) |
| Total cholesterol (mg/dl) | 186.5 (102.0–332.1) |
| AFP (ng/ml) | 2.4 (0.8–303.6) |
| Cirrhosis (−/+)[b] | 341/40 |
| Hepatocarcinogenesis (+/−) | 17/364 |

F, female; M, male; BMI, body mass index; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase: AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; ALP, alkaline phosphatase; AFP, alpha-fetoprotein.
Values are expressed as median (range).
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound findings.

TABLE II. Factors Associated With Hepatocarcinogenesis (Univariate Analysis)

| | Hazard ratio (95% CI) | *P*-value |
|---|---|---|
| Sex | | |
| F | 1 | |
| M | 8.282 (1.892–36.259) | 0.005 |
| HBV-DNA (log copies/ml) | | |
| ≤5.0 | 1 | |
| >5.0 | 7.133 (2.699–18.852) | <0.001 |
| Persistently normal ALT[a] | | |
| Presence | 1 | |
| Absence | 3.939 (1.126–13.776) | 0.032 |
| AST (IU/L) | | |
| ≤40 | 1 | |
| >40 | 4.046 (1.157–14.140) | 0.029 |
| Platelets ($\times 10^4$/m$^3$) | | |
| ≥15 | 1 | |
| <15 | 7.961 (2.922–21.690) | <0.001 |
| Cholinesterase (IU/L) | | |
| ≥431 | 1 | |
| <431 | 4.865 (1.368–17.298) | 0.015 |
| Albumin (g/dl) | | |
| ≥3.5 | 1 | |
| <3.5 | 8.086 (2.567–25.474) | <0.001 |
| Total cholesterol (mg/dl) | | |
| ≥130 | 1 | |
| <130 | 9.704 (2.740–34.367) | <0.001 |
| AFP (ng/ml) | | |
| ≤10 | 1 | |
| >10 | 6.779 (1.445–31.809) | 0.015 |
| Cirrhosis[b] | | |
| Absence | 1 | |
| Presence | 18.033 (6.6055–19.233) | <0.001 |

W, female; M, male; HBV, hepatitis B virus; AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; AFP, alpha-fetoprotein. *P*-values and hazard ratio were calculated by Cox proportional hazard model.
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound.

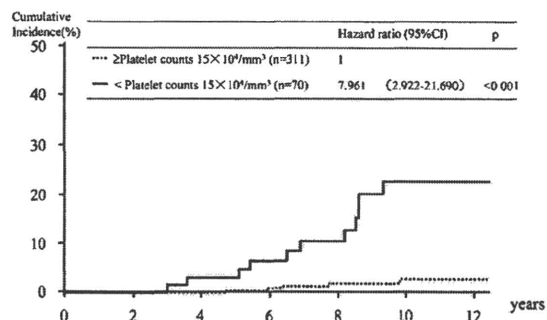


Fig. 4. Incidence of HCC according to platelet counts. The 5- and 10-year cumulative incidences of HCC was 0.4% and 2.6%, respectively, in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311), and 2.9% and 22.9% in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70). The cumulative incidence of HCC was significantly higher in the latter group than in the former.

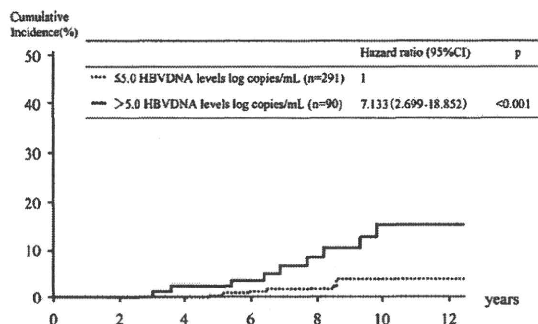


Fig. 5. Incidence of HCC according to serum HBV-DNA levels. The 5- and 10-year cumulative incidences of HCC was 0.4% and 3.7%, respectively, in patients with HBV-DNA levels <5.0 log copies/ml (n = 291) and 2.3% and 15.5%, respectively, in patients with HBV-DNA levels ≥5.0 log copies/ml (n = 90). The cumulative incidence of HCC was significantly higher in the latter group than in the former.

of cirrhosis were highest in Group D (Table IV). Group D showed the highest rate of incidence of HCC, followed by Groups B and C, as compared with Group A (Fig. 6).

## DISCUSSION

The current studies revealed that the risk of developing HCC increases with decreasing platelet counts, decreasing total cholesterol levels, and increasing HBV-DNA levels in patients with average ALT integration values ≤40 IU/L.

ALT, AST, gamma-GTP, ALP, and AFP levels fluctuated within individual patients. Therefore, repeated measurements of these tests are important for accurate interpretation of the data. The arithmetic mean value is often used in the measurement of these tests; however, this value can be greatly affected by the period of time between measurements. Therefore, integral calculus was used to determine the value of these markers. Because this determination is strongly affected by the follow-up period, the average integration value was divided by the time of follow-up. The average integration

TABLE III. Multivariate Analysis of Factors Associated With Development of Hepatocellular Carcinoma

| Factor | Hazard ratio (95% CI) | $P$-value |
|---|---|---|
| Sex | | |
| F | 1 | |
| M | 6.011 (1.353–26.710) | 0.018 |
| HBV-DNA (log copies/ml) | | |
| ≤5.0 | 1 | |
| >5.0 | 5.125 (1.880–13.973) | 0.001 |
| Platelets ($\times 10^4$/mm$^3$) | | |
| ≥15 | 1 | |
| <15 | 4.803 (1.690–13.647) | 0.003 |
| Total cholesterol (mg/dl) | | |
| ≥130 | 1 | |
| <130 | 5.983 (1.558–22.979) | 0.009 |

F, female; M, male; HBV, hepatitis B virus.
$P$-values and hazard ratios were calculated using the Cox proportional hazard model.

value is more meaningful than the arithmetic mean value [Kumada et al., 2007].

In the present study, there was no difference between patients with average ALT integration values of 0–20 IU/L versus those with 21–40 IU/L. Thus, ALT levels are not good predictors of HCC development in patients with hepatitis B, as opposed to hepatitis C [Yuen et al., 2005; Sherman, 2005]. Furthermore, the change pattern of ALT was evaluated in the persistently normal ALT group and the intermittently normal ALT group. The results of the univariate analysis suggest that intermittently normal ALT levels, high AST levels, low cholinesterase levels, low albumin levels, and high AFP levels are associated significantly with HCC development; however, not all of these factors were significant in the multivariate analysis.

HBV-DNA levels at the start of the follow-up period correlated with the cumulative incidence of HCC. Chen et al. [2006] reported the adjusted hazard ratios for HCC development in HBeAg-seronegative subjects with normal ALT levels. Compared with participants in whom serum HBV-DNA levels were <300 copies/ml, the adjusted hazard ratio for developing HCC was 1.3 (95% confidence interval, 0.5–3.2; $P = 0.05$) for participants with serum HBV-DNA levels of 300–9,999 copies/ml; 2.7 (1.2–6.3; $P = 0.02$) for levels of 10,000–99,999 copies/ml; 7.2 (3.2–16.6; $P < 0.001$) for levels of 100,000–999,999 copies/ml; and 14.3 (6.2–32.8; $P < 0.001$) for levels of 1 million copies/ml and greater. It is emphasized that the cumulative incidence of HCC increases in patients with increased HBV-DNA levels, even if patients have normal ALT levels.

Lok and McMahon [2004] reported that HBV-DNA levels $>10^5$ copies/ml should be considered clinically significant. Their recommendation is supported by a meta-analysis of 26 trials of anti-HBV therapy which evaluated the association between viral load and hepatic inflammatory activity, as determined by hepatic histology and aminotransferase activity [Mommeja-Marin et al., 2003]. Thus, it is important for patients to maintain low HBV-DNA levels (i.e., $\leq 10^5$ copies/ml). These findings suggest that effective control of HBV replication, indicated by a decrease in serum HBV-DNA levels following antiviral therapy, may reduce the ultimate risk of developing HCC. Furthermore, it is believed that treatment with nucleosides or nucleotide analogues will decrease the cumulative incidence of HCC [Liaw et al., 2004; Piao et al., 2005].

The present study reveals that a low platelet count is a predictive factor for the development of HCC. Cirrhosis is an established risk factor for HCC in patients with HBV [Liaw et al., 1989; McMahon et al., 2001; Yu et al., 2002; Murata et al., 2005]. Ultrasonography produces detailed cross-sectional images of the liver and its surrounding structures. To distinguish cirrhosis patients from non-cirrhosis patients was attempted according to typical ultrasound findings [Caturelli et al., 2003; Iacobellis et al., 2005; Shen et al., 2006]. The presence of cirrhosis diagnosed by ultrasonography

TABLE IV. Patients Characteristics, According to HBVDNA Levels and Platelet Counts

| HBV-DNA (log copies/ml)<br>Platelets ($\times 10^4/m^3$) | Group A<br>$\leq 5.0$<br>$\geq 15 \times 10^4$ (n = 257) | Group B<br>$\leq 5.0$<br>$< 15 \times 10^4$ (n = 45) | Group C<br>$> 5.0$<br>$\geq 15 \times 10^4$ (n = 54) | Group D<br>$> 5.0$<br>$< 15 \times 10^4$ (n = 25) |
|---|---|---|---|---|
| Age (years) | 49 (12–84) | 51 (24–75) | 47 (15–73) | 52 (33–82) |
| Sex (F/M) | 136/121 | 25/20 | 29/25 | 11/14 |
| BMI ($kg/m^2$) | 22.6 (14–36.3) | 22.5 (16–28.2) | 22.2 (16.7–32.4) | 20.9 (16.9–36.4) |
| HBV genotype (A/B/C/D) | 7/20/88/2 | 0/1/20/0 | 1/3/26/0 | 0/0/15/0 |
| HBeAg (positive/negative)*** | 5/252 | 3/42 | 36/18 | 15/10 |
| ALT (IU/L)*** | 19.7 (8.7–39.1) | 25.3 (11.2–38.2) | 29.8 (12.2–39.9) | 32.1 (18.3–38.4) |
| Persistently normal ALT (+/−)[a],*** | 153/104 | 14/31 | 14/40 | 1/24 |
| Total cholesterol (mg/dl)*** | 191.5 (114–332.1) | 169.1 (102–259.2) | 190.1 (147.1–254.4) | 165.5 (112–234) |
| AFP (ng/ml)**** | 2.2 (0.8–119.8) | 2.6 (0.8–20.8) | 2.8 (0.8–45.5) | 4.7 (1.1–303.6) |
| Cirrhosis (−/+)[b],*** | 253/4 | 27/18 | 50/4 | 11/14 |
| Hepatocelluar carcinoma (+/−)*** | 2/255 | 5/40 | 4/50 | 6/19 |

F, female; M, male; BMI, body mass index; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; AFP, alpha-fetoprotein.
*P*-values were calculated using the Kruskal–Wallis test or the chi-square test. Values are expressed as median (range).
[a]Persistently normal ALT values includes patients with $\leq 40$ IU/L.
[b]Cirrhosis diagnosed by ultrasound findings.
***$P < 0.0001$.
****$P < 0.0005$.

was strongly associated with the increased incidence of HCC by univariate analysis. Anatomical constraints and interobserver variability, however, remain limiting factors. In this study, histological confirmation was obtained in only 20 patients (6.3%). It is thought that this study had limitations because the liver histology was not obtained in many cases. Liver biopsy is still the "gold standard" for assessing liver fibrosis; however, it is not practical to undertake biopsies on all patients because of the potential complications which might arise from this procedure. Furthermore, results often differ depending on the pathologist, and results for liver fibrosis in liver biopsy specimens do not always reflect the grade of fibrosis in the entire liver. In contrast, the platelet count is a useful surrogate marker for the diagnosis of cirrhosis. Lu et al. [2006] reported that the best cutoff platelet count for a diagnosis of cirrhosis is $15.0 \times 10^4/mm^3$. The primary aim of this study was to identify serological markers associated with the development of HCC. Because of this, cirrhosis diagnosed by ultrasonography was excluded from the multivariate analysis. On the other hand, a low cholesterol level is associated with hepatocarcinogenesis, too. Hypocholesterolemia is found frequently in advanced liver disease because the liver is the most active site of cholesterol metabolism [D'Arienzo et al., 1998]. Four of 12 patients (33.3%) with <130 mg/dl serum total cholesterol developed HCC during follow-up period. It seemed that low platelet counts and hypocholesterolemia were confounding factors for identifying cirrhosis. Platelet counts were used as a parameter for cirrhosis in this study.

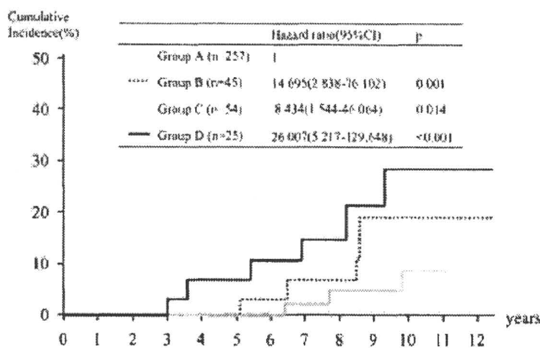


Fig. 6. The cumulative incidence of HCC according to HBV-DNA levels and platelet counts. HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts $< 15.0 \times 10^4/mm^3$ [n = 45]; C: HBV-DNA levels $\geq 5.0$ log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 54]; and D: HBV-DNA levels $\geq 5.0$ log copies/ml and platelet counts $< 15.0 \times 10^4/mm^3$ [n = 25]). Group D had the highest incidence rate of HCC (26.007 [5.217–129.648], $P < 0.001$), followed by Group B (14.695 [2.838–76.102], $P = 0.001$) and Group C (8.434 [1.544–46.064], $P = 0.014$), as compared with Group A.

The HBV genotype is also predictive of the development of HCC [Chan et al., 2004; Yu et al., 2005]. In Japan, HBV genotype C is the predominant genotype [Orito et al., 2001]. Genotype C is associated with higher HBV-DNA levels and a greater risk of HCC than genotype B [Chan et al., 2004]. In the present study, 149 of 183 patients (81.4%) were infected with HBV genotype C. All eight patients with HCC in whom HBV genotype was determined were infected with genotype C. It was difficult to evaluate the relationship between HBV genotype and incidence of HCC in this study.

This study has some limitations such as the potential for selection bias due to a retrospective analysis of a cohort of patients. Therefore, an effort was made to minimize the influence of bias by using average integration values of various biochemical markers and a multivariate analysis.

In conclusion, high HBV-DNA levels and low platelet counts are associated with an increased incidence of HCC in patients infected with hepatitis B who have normal ALT values. Therefore, maintenance of low HBV-DNA levels is important for the prevention for

**HCC in patients with low platelet counts, even when the ALT values fall within the current normal range.**

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***Original Article***

# Evaluation for clinical utility of GPC3, measured by a commercially available ELISA kit with Glypican-3 (GPC3) antibody, as a serological and histological marker for hepatocellular carcinoma

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*Aims:* We evaluated the clinical utility of glypican-3 (GPC3), which has been proposed as a potential novel tumor marker for hepatocellular carcinoma (HCC), as a serological and histological marker for HCC.

*Methods:* The serum GPC3 level was compared between 200 patients with HCC and 200 patients with chronic liver disease (CLD). In addition, the expression of GPC3 was examined with immunohistochemistry on 38 resected specimens from patients with HCC. A commercially available GPC3 antibody was used for these analyses.

*Results:* The median values of serum GPC3 in patients with HCC and with CLD were 924.8 pg/mL and 1161.6 pg/mL, respectively. We found no elevation of serum GPC3 level in patients with HCC in comparison with those with CLD; rather the level was higher in patients with CLD ($P < 0.0001$). In immunohistochemical analysis, 14 of 38 (36.9%) HCC tissues were positive for GPC3, whereas no corresponding non-cancerous tissue was positive. The positivity for GPC3 tended to increase with pathologic decreased differentiation of HCC.

*Conclusions:* We did not find serum GPC3 level, measured by a commercially available ELISA kit with GPC3 antibody, to be useful in the diagnosis of HCC. However, we did observe increased GPC3 staining in HCC tissue with moderate or poor differentiation, suggesting that GPC3 is produced by HCC tumors. This lack of utility could have been due to the measuring procedure used in the present study. Further evaluation of GPC3 in HCC with other measuring procedures is needed.

**Key words:** ELISA, glypican-3, hepatocellular carcinoma, immunohistochemistry, tumor marker

## INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most prevalent malignancies worldwide. It is the sixth most common cancer, and the third most common cause of cancer-related death, in the world.[1] In Japan, HCC is the third most common cause of death from cancer in men, and the fifth most common in women.[2] The most important risk factor for the development of HCC is liver cirrhosis, regardless of etiology.[3] In addition, chronic infection with hepatitis viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV), as well as high alcohol intake, increase the risk of HCC.[4–7]

Alpha-fetoprotein (AFP),[8–11] Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3),[12–14] and des-gamma-carboxy prothrombin (DCP)[15–17] have been reported to be useful as serological tumor marker for HCC in cases of HCC surveillance and diagnosis, and in the evaluation of patient prognosis.[18] Nevertheless, all tumor markers have limitations and therefore the identification of additional tumor markers for HCC with high sensitivity and specificity is necessary.

Glypican-3 (GPC3) is a member of the glypican family of glycosyl-phosphatidylinositol-anchored cell-

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